National Institute of Allergy and Infectious Diseases

WORKSHOP SUMMARY
IMAGING TECHNOLOGY AND STUDY OF IMMUNE FUNCTION

April 8-9, 2003

6700B Rockledge Drive
Bethesda, Maryland

Summary

On April 8-9, 2003 the National Institute of Allergy and Infectious Diseases (NIAID) convened a workshop on Imaging Technology and the Study of Immune Function. The objectives of the meeting were twofold: (1) to discuss progress on programs funded under RFA AI 99-004, New Imaging Technologies for Autoimmune Diseases; and (2) to advise the NIH on future directions and areas of opportunity that imaging technology can bring to advance our understanding of immune system function.

Speakers

William Duncan, Deputy Director of the Division of Allergy, Immunology and Transplantation, NIAID, provided opening remarks on the role of imaging technology in the investigation of immunological diseases, vaccine and therapeutic drug efficacy evaluation, and immune cell localization. He also discussed the importance of collaborative research efforts and the multiple institutes that support this research.

Timothy Gondré-Lewis, Program Officer in the NIAID, Division of Allergy, Immunology and Transplantation, provided an overview of the meeting in the context of the goals of the RFA and its design: to bring together experts in imaging, contrast agent design and synthesis, and immunology to evaluate the *in vivo* function of immune cells in normal and abnormal immune responses.

John Mountz – University of Alabama
Development of Tracers and Mouse Models for T cell Imaging

To obtain *in vivo* nuclear images of autoreactive T cells, there are four major challenges that must be overcome: define or introduce a high affinity receptor; have the capacity for multi-image acquisition; minimize background binding in nearby organs to obtain clear images of organs of interest; and maintain cell viability for *in vivo* imaging. To address these challenges, Dr. Mountz developed a triple transgenic mouse. First, mice were made transgenic for the human coxsackie-adenovirus receptor (hCAR) molecule under control of a ubiquitin or CD2 promoter. This receptor increases the efficiency of transfection using adenovirus vectors. CD2-hCAR transgenic mice were transfected with an adenovirus vector bearing the human somatotropin receptor (hSSTR2) linked to the green fluorescent protein (GFP). The hSSTR2 enables the investigator to increase the specificity of tracer

uptake by utilizing a somatostatin peptide analogue, NeoTect® (Berlex Laboratories, Inc). GFP was used in order to determine transfection efficiency by immunofluorescent microscopy. The transfection efficiency was about 80 percent, when the adenovirus vector was used at 50 plaque forming units (PFU) per cell. The hCAR/hSSTR2 transgenic mouse was then crossed to D^b/H-Y TCR transgenic mice. The presence of the H-Y antigen makes T cells transferred into non-transgenic D^b male mice autoreactive, and allowed *in vitro* and *in vivo* examination of activation and activation-induced cell death in reactive T cells. Dr. Mountz also noted that stability of the hCAR transgene allows for the incorporation of other novel receptors that can be used as targets for radiolabelled tracers and *in vivo* nuclear imaging.

This transfection protocol could result in the development of a number of novel animal models for the analysis of *in vivo* T cell activation in autoimmune diseases.

Chien Ho – Carnegie Mellon University
Non-Invasive Detection by MRI of Immune Cell Accumulation at the Rejection Graft
To detect allograft rejection, Dr. Ho devised a two-pronged approach that examines immune cell infiltration and organ dysfunction. Immune cell infiltration was detected by use of magnetic resonance contrast agents composed of ultra small paramagnetic iron oxide (USPIO) particles that are readily internalized by phagocytic cells, but not T cells. Tat (transactivator) peptide from HIV and dextran were coated on cross-linked small paramagnetic iron oxide particles (CL-SPIO-Tat). Molecules of fluorescein isothiocyanate (FITC) were linked to these particles. The presence of the Tat peptide increased the specificity of the particles for T cells. Organ dysfunction was assessed by functional imaging of the allograft at various time points after transplantation. This methodology was successfully applied in kidney, lung, and heart transplantation rodent models.

After organ transplantation, infused CL-SPIO-Tat-FITC particles were rapidly and specifically detected in T cells and macrophages. Serial magnetic resonance imaging (MRI) showed accumulation of transferred *in vitro* labeled T cells at the site on day four post-transplant kidney in a rat model. The accumulation of cells at the rejection site was seen only in allograft kidneys, not in isografts, and correlated directly with the pathological rejection grade of the graft. Furthermore, clear differences were detected in kidney function by first-pass renal images, and analysis of signal intensity between rejected and non-rejected kidneys following perfusion of CL-SPIO particles four days post-transplant. These results suggest that MRI-based multiparametric examination might be a useful tool to monitor transplant function or dysfunction through non-invasive diagnostic means following transplant.

Jennifer Allport – Massachusetts General Hospital - Harvard Medical School
***In vivo* High Resolution 3D Imaging of Antigen-Specific Cytotoxic T-Lymphocyte Trafficking**
T cell trafficking and recruitment to tumors *in vivo* was examined by MRI coupled with the use of modified superparamagnetic particles (cross-linked iron oxide (CLIO)). The particle modification included the addition of multiple copies (>20) of the HIV Tat peptide to create a CLIO-HD (highly derivitized) particle. The addition of multiple copies of the Tat peptide led to greater T cell uptake. Uptake of the particles by T cells was proportional to the amount of Tat peptide per particle and the time of incubation *in vitro*. The CLIO-HD particle was not toxic to the T cells, and did not induce proliferation or cytotoxicity. Labeled T cells also exhibited no change in their ability to interact with endothelium or chemotax in response to stimuli.

To assess the *in vivo* tracking of T cells to tumor, the B16-OVA melanoma tumor expressing the SIINFEKL-peptide of chicken ovalbumin was injected into mice. After three days growth *ex vivo,* CLIO-HD labeled, OVA-specific, OT-1 $CD8^+$ T cells were injected into tumor bearing hosts. MRI showed the rapid recruitment of $CD8^+$ T cells to the tumor site (<12h), and was the first high-resolution imaging of T cell recruitment to intact tumors in live mice. The investigators were able to detect between 3 and 60 cells per voxel[1] *in vivo*. Significant differences were also observed in the distribution of T cells in the tumor based on delivery methods. Dr. Allport examined the effects of injecting equal numbers of T cells either as a bolus injection or serial injection. When injected serially, T cells were distributed to different regions of the tumor. Furthermore, only the serial administration of T cells resulted in tumor regression, suggesting that this model of T cell-based therapy may be clinically useful, particularly when combined with *in vivo* visualization techniques to monitor tumor regression.

[1] voxel- volume pixel, the smallest distinguishable box shaped part of a three-dimensional image.

Ronald Germain – National Institute of Allergy and Infectious Diseases
High Resolution 4D Imaging of Immune Cell Interactions, Protein Distribution, and Signaling in Lymphoid Tissues

To understand the interface between the innate and adaptive immune systems, Dr. Germain utilized high-resolution spatiotemporal imaging of the T cell-dendritic cell interface. The system involved the adoptive transfer of antigen-specific T cells to an antigen-challenged host. Antigen-bearing dendritic cells from an antigen-challenged host and transferred T cells were recovered from intact draining lymph nodes. Explanted lymph nodes were microscopically visualized by laser-scanning confocal microscopy or by multiphoton microscopy to address the long-standing immunological question of "how long do antigen bearing dendritic cells and T cells associate in the lymph nodes?"

The majority of T cells showed monogamous associations with dendritic cells, some associations were reported to last for 36 hours. These findings are in contrast to reports of other investigators, which showed vigorous association and migration of T cells with multiple dendritic cells. These differences could be attributed to several key experimental differences between the researchers. For example, subscapular T cells, within lymph nodes, are less motile than deeper cortex T cells. It was also noted that there are differences in the movement of $CD4^+$ and $CD8^+$ T cells. Furthermore, the level of cellular oxygenation and method of animal sacrifice played important roles in the migratory function of T cells in explanted lymph nodes.

The data suggested that there are clear morphological signs of T cell activation after 36 hours of dendritic cell contact. The T cells detached from the dendritic cells, sometimes in conjunction with cell division. Future investigations will include *in vivo* analysis of $CD4^+$ and $CD8^+$ T cell interaction with the same dendritic cell, and the interaction of $CD4^+$ and $CD8^+$ T cells with each other, in exclusion of dendritic cells. Other studies will examine mature T cells at peripheral sites of antigen encounter, movement of infectious agents in epithelial and lymphoid sites, and the activation and localization of other immune effector cells, such as NK and NKT cells.

James Rosenbaum – Oregon Health Sciences University
Using the Eye to Image the Immune Response

The eye is often the target for immune-mediated reactions and disease. Ocular immunology has some unique disadvantages including anatomical sequestration and the possible lack of lymphatics, both of which may limit immune cell trafficking. The eye also provides unique advantages for imaging immune responses. Taking advantage of the transparency of the cornea, Dr. Rosenbaum is

able to photograph and document cell migration in the anterior segment of the murine eye, using a slit-beam epifluorescent microscope and time-lapse videography. Cells can be labeled *in vivo* or *ex vivo* and antigen can be injected directly into the aqueous humor without surgical trauma to the animal. In the absence of infection, the aqueous humor is devoid of cells. In an inflammatory reaction, leukocytes are seen in the aqueous humor and anterior chamber of the eye.

Several novel observations have been made using this system, some of which affirm the unique nature of immune responses in ocular tissues. Antigen encounter in the periphery results in a great deal of movement on the part of the antigen-presenting cell, including migration to regional lymph nodes. However, after antigen introduction, antigen presenting cells in the iris tend to remain stationary. In contrast, neutrophils attracted to the iris stroma by antigen are nearly continuously in motion. The mapped motion of the neutrophils in the iris is extremely complex and seemingly random, though the cells seldom bump into each other.

To visualize T cell-antigen presenting cell interaction *in vivo*, a T cell-mediated uvetis model was used in which antigen and antigen-specific T cells were injected into the eye. There appeared to be a plane upon which the T cells moved in the field of the ciliary body of the iris, with the T cells displaying a focal distribution pattern. This finding suggested T cells may use zonular fibers within the ciliary body for migration. T cell activation was detected *in vivo*, in an IL-2/GFP knock-in mouse model. In this model, IL-2 production by activated T cells led to synthesis of GFP under control of the IL-2 promoter, and visualization of activated T cells.

Imaging of immune responses in the eye can be done either in real time or with time-lapse video. These techniques may become applicable to non-invasive *in vivo* examination of ocular sites of inflammation in humans, for conditions such as schleritis or anterior chamber-associated immune deviation.

Donald Kimpel – Louisiana State University
Epifluorescent Videomicroscopy in Inflammatory Arthritis
Rheumatoid arthritis is the most common form of inflammatory arthritis, affecting 1% of the population worldwide. This condition can affect multiple joints, cause bone erosion, and lead to increased morbidity. Despite extensive research, the antigen and the inflammatory trigger remain unknown. In recent work from the laboratory of Dr. Donald Kimpel, leukocyte trafficking was examined as means of evaluating potential therapeutic targets for this inflammatory disorder.

Peptidoglycan-polysaccharide (PGPS) derived from *Streptococcal* cell wall was used to induce arthritis in female Lewis rats. The early acute phase of the inflammatory reaction is characterized by macrophage activation. This phase is followed by a chronic phase of inflammation, which is T cell dependent. At this stage, there is a two- to three-fold increase in the number of adherent leukocytes in inflamed joints. Using a dual-radiolabeled monoclonal antibody, Dr. Kimpel observed systemic and local changes in adhesion molecule expression. ICAM-1, VCAM-1, and P-selectin were increased significantly in joints. These localized increases in adhesion molecule expression caused increased leukocyte trafficking to and adhesion in the affected joints. Increased leukocyte adhesion in non-articular vascular beds was also observed. The cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) are known to mediate inflammatory arthritis. These cytokines also were detected at significantly higher levels in blood plasma in the peptidoglycan-polysaccharide arthritis model compared to normal animal.

Future experiments from this research team will assess the source of TNF-α and IL-1β: endothelium, leukocytes, or both. Furthermore, there are plans to evaluate the effect of blocking specific adhesion molecules on leukocyte adhesion and arthritis development. This assessment of the local and systemic nature of inflammatory arthritis may uncover its mechanism of generation and provide targets to reduce arthritis-associated morbidity.

Henry Kaplan – Washington University School of Medicine
***In vivo* Monitoring of T Cell Distribution Using ^{64}Cu-DOTA-CD4^{+} Monoclonal Antibody in Rodents with a Dedicated MicroPET Scanner**
Imaging organs and tissues by positron emission tomography (PET) provides valuable information about aspects of tissue morphology and function. To follow T cell migration *in vivo*, Dr. Kaplan conjugated a CD4 molecule-specific antibody to ^{64}Cu radiolabeled 1,4,7,10-tetraazacyclododecane-N, N',N",N"'-tetraaacetic acid (DOTA). The ^{64}Cu-DOTA-CD4^{+} mAb was used to label T cells *ex vivo* to test uptake, washout, and cell viability. Importantly, labeling did not interfere with T cell viability. *Ex vivo* labeling of T cells followed by injection allowed the investigators to monitor T cell trafficking and circulation in the animal. The distribution of T cells in a normal animal initially showed highest concentrations in the liver and spleen. After 24 hours, T cells were primarily localized to secondary lymphoid tissue (spleen and lymph nodes). ^{64}Cu-DOTA-CD4^{+} mAb was also injected, *in vivo*, for assessment of T cell bio-distribution. Similar results were obtained with *in vivo* and *ex vivo* labeled T cells.

The number of T cell receptors per cell and the least number of T cells required for detection by PET were determined by the use of radioisotope labeled T cells coupled to PET. The lower limit of detection was 160 CD4^{+} T cells. PET and ^{64}Cu-DOTA-CD4^{+} mAb were used to study experimental Acute Anterior Uveitis (AAU) in the rat. AAU is an autoimmune disease mediated by CD4^{+} T cells. ^{64}Cu-DOTA-CD4^{+} mAb was used to monitor T cell distribution during the course of disease and following disease resolution. During disease progression (day 14), there was a decrease in T cell populations in distal abdominal lymphatics and an increase in head and neck lymph nodes. After disease resolution, the CD4^{+} T cell pool in the head and neck lymph nodes returned to normal levels. These data suggested that commercially available imaging devices such as microCT, microPET, SPECT, and optical imaging may provide valuable insight into the pathophysiology of autoimmune disease in rat and other animal models.

David Reichert – Washington University School of Medicine
MicroPET Imaging of Rheumatoid Arthritis in the K/BxN Murine Model
The K/BxN murine model of rheumatoid arthritis shares many of the pathological features of human rheumatoid arthritis, including histological features such as: leukocyte invasion, synovitis, pannus formation, and cartilage and bone destruction. The K/BxN mouse produces autoantibodies to the ubiquitously expressed glycolytic enzyme glucose-6-phosphate isomerase (GPI) and develops joint inflammation by 4-5 weeks of age. Ubiquitous expression of GPI and systemic circulation of the GPI-autoantibody in the K/BxN mouse seems incongruous with tissue-specific activity.

To address this question, Dr. Reichert conjugated GPI-specific IgG to a DOTA linker and labeled the conjugate with ^{64}Cu. Using ^{64}Cu-DOTA-GPI polyclonal antibodies injected into naïve animals, he tracked antibody localization and deposition. Within twenty minutes, antibodies accumulated at

the distal joints of the front and rear limbs. Localization in the joints was observed for 24 hours, and correlated with the onset of inflammation.

To determine the *in vivo* trigger for the localization and inflammatory events, a series of experiments were performed to identify the cells and cellular mechanisms involved. Potential mechanisms included: direct trafficking of the antibodies to the joints, complement-mediated activation events, or Fc-R-dependent activation. Using mice that are anti-GPI-induced arthritis resistant ($Fc\gamma RI^{-/-}$, $FcR\gamma RIII^{-/-}$, $C5^{-/-}$, $TNF\alpha^{-/-}$, mast cell$^{-/-}$, and neutrophil$^{-/-}$ mice), Dr. Reichert's team sought to determine if anti-GPI antibodies localized in the joints. In the absence of FcR, mast cells, and neutrophils, very little antibody was deposited in the joints. However, complement component C3 and C5 deficient mice showed high levels of antibody deposition. Injection of anti-type II collagen IgG, as a control in the complement component deficient mice, does not lead to a similar pattern of accumulation at the joint spaces. However, if preformed immune complexes were injected there was a significant accumulation of antibody at the joint spaces of these mice.

These results suggest the following five step model for joint-specific localization of autoantibody: (1) immune complexes are bound by an FcγRIII-expressing neutrophil in a synovial vessel; (2) soluble factors are released by the neutrophils and result in increased vascular permeability and extravasation of immune complexes; (3) mast cells bind immune complexes in the extravascular space; (4) mast cell degranulation leads to increased vascular permeability and influx of inflammatory cells, anti-GPI antibodies, and complement components; and (5) these factors trigger pro-inflammatory pathways leading to the destruction of cartilage and development of rheumatoid arthritis. Dr. Reichert's future research direction includes determining the importance of antibody-specificity on joint retention, the source and type of soluble mediators responsible for changes in vascular permeability, and the role of FcR on different cell types.

Thomas Meade – California Institute of Technology
Biochemically Activated MR Contrast Agents

Magnetic resonance imaging (MRI) is a versatile, sensitive, and powerful diagnostic tool, and is preferred for diagnosing most diseases of the brain and central nervous system because it is a non-invasive and relatively quick procedure. The addition of MR contrast agents can improve the sensitivity and/or specificity of images. Dr. Meade's work focuses on the design of targeted and activatable MR contrast agents for neuroimaging and lymphocyte study.

One of the challenges to developing new MR agents for neuroimaging is crossing the blood-brain barrier. To address this challenge, contrast agents are being designed using small molecule transporters or drug-transporters, such as Ru-486 linked to Gd^{3+}. Additional work is ongoing to explore the potential to follow cell migration and division in stem cell and embryonic cell populations of mice.

Other work focuses on the production of MR agents that are biochemically activated in a particular environment. For example, an MR agent (e.g.; DOTA) with bound gadolinium (Gd^{3+}-a T_1 enhancing agent) is protected by a dendrimer (tree-like polymer). Cleavage of the dendrimer exposed Gd^{3+} to water, which activates the Gd^{3+} and allows detection by MR. The structure of Gd^{3+}, which includes seven unpaired electrons, endows it with the ability to rapidly exchange electrons with water. This exchange produces a randomly fluctuating magnetic field in its vicinity. The T_1 relaxation efficiency is increased and the difference in field strength is detectable in the

image. Further exploration of agents that are activatable *in vivo* either by enzymatic processing or by reversible binding to an intracellular messenger are areas of study by the Meade laboratory.

Recommendations to NIH: Future Opportunities and Directions

1. Reissue the RFA: New Imaging Technologies for Autoimmune Disease. The RFA was successful in bringing together interdisciplinary scientists to work on imaging immune function. In order to realize the full potential of the technology, there needs to be continued financial commitment. The group believed that recompeting in a revised RFA would be more effective than funding individual R01 applications for two reasons. First, applications would undergo a better review by a single scientific review group familiar with imaging modalities and their applications to immunology. Second, the participation in the currently funded RFA has proven that a dedicated imaging program can foster collaborations and information exchange among participants.

2. Techniques:

A. MicroPET
   - Identify better ways of attenuating the signal to detect structures that currently are overwhelmed by background noise.
   - Include multi-isotope recordings in functional studies.
   - Develop of antigen specificity (currently the system is antigen non-specific).

B. MRI
   - Improve the sensitivity and specificity of the magnetic field.
   - Improve post-data processing to obtain information from the images more efficiently.
   - Develop contrast agents that provide a better approximation of human disease in animal models, thus greatly enhancing the use of MR for disease visualization.

C. Contrast Agent Development
   - Develop contrast agents that broaden our understanding of the chemistry of cellular function, not just anatomical location and cellular movement.
   - Obtain approvals to conduct *in vivo* assessments of agents in humans.

D. Single Cell Imaging
   - Identify limitations to single cell imaging, *in vivo*.
   - Advance technology to allow for shorter imaging time, greater resolution, and enhanced real-time image collection.
   - Improve computational aspects of imaging.

E. Instrumentation
   - Develop post-imaging processing tools to obtain greater amounts of information, more rapidly, from images.
   - Provide processing tools that are universally available and provide greater cross talk across imaging modalities.